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Analysis of *DRB1* exon 2 genotyping by STR size analysis in Suffolk and Texel sheep breeds

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Alleles of the *DRB1* exon 2 locus of the major histocompatibility complex have recently been associated with genetic resistance to gastrointestinal nematodes in sheep. While sequence-based typing is the standard method for allele discrimination, a rapid, high throughput method for *DRB1* exon 2 genotyping is required if such information is to be incorporated into national breeding programmes. Previous studies have highlighted a simple tandem repeat (STR) located within intron 2 of the *DRB1* gene, which could potentially be used to accurately assess the allele present within the adjacent exon 2. The aims of this study were firstly to compare two methods of STR analysis, Genescan™ and autoradiography, and secondly to investigate if STR analysis of *DRB1* intron 2 could be used to accurately assess the profile of *DRB1* exon 2. Six *DRB1* exon 2 alleles were identified by sequence-based typing in Suffolk (n = 31) and eight in Texel (n = 60) sheep. The results indicated that Genescan™ was a more accurate method of STR analysis than autoradiography. The expected 1:1 correspondence between STR size, analysed by Genescan™ and *DRB1* exon 2 allele, determined by sequence-based typing, was not observed. However, the correspondence was found to be degenerate, whereby some alleles were associated with two STR sizes. Thus, irrespective of the STR size identified, STR analysis by Genescan™ identified the correct allele in all cases within both populations of animals studied. However, the Genescan™ method of allele identification cannot be used for Suffolk × Texel crossbred progeny or in other breeds where the relationship between STR size and *DRB1* exon 2 allele is not known.

Keywords: Autoradiography; *DRB1* exon 2; Genescan; linkage disequilibrium; simple tandem repeat

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Introduction

There is substantial evidence supporting inter- and intra-breed differences in resistance to gastrointestinal nematode infection in sheep (Miller *et al*., 1998; Hanrahan and Crowley, 1999; Matika *et al*., 2003). A key aim of research on resistance to nematodes is to identify the genes that confer resistance in order to facilitate breeding programmes to select for increased resistance. Considering the central role the major histocompatibility complex (MHC) plays in the immune system, it is not surprising that the *DRB* region remains an attractive candidate for disease association studies. In general, associations between the MHC-*DRB1* gene and disease resistance are focused primarily on the second exon because of its high level of polymorphism and functional significance in binding and presenting foreign antigen. Over 80 *DRB1* exon 2 alleles have been identified in sheep, two of which have been associated with decreased resistance (Sayers *et al*., 2005) and one with increased resistance to nematode infection (Sayers *et al*., 2005; Schwaiger *et al*., 1995). If selection to reduce the impact of nematode infection is to be implemented a rapid method of *DRB1* exon 2 genotyping is required. A number of different methods have been employed to differentiate between *DRB1* exon 2 alleles; immunoprinting by autoradiography (Schwaiger *et al*., 1993), cloning followed by sequencing (Konnai *et al*., 2003a), PCR-restriction fragment length polymorphism (Blattman *et al*., 1993; Grain *et al*., 1993; Konnai *et al*., 2003b), single-stranded conformational polymorphism (Kostia *et al*., 1998; Outteridge *et al*., 1996) and sequence-based typing (Sayers *et al*., 2005).

Above all others, the immunoprinting technique is popular because it has the advantages of simple differentiation of alleles of heterozygous individuals and speed, and has been used extensively and successfully in disease association studies in both humans (Epplen *et al*., 1995, 1997) and animals (Schwaiger *et al*., 1995). The principle of this technique, investigated in a number of different animal species by Schwaiger *et al*. (1993), is that there is complete linkage disequilibrium between the Simple Tandem Repeat (STR), a microsatellite located within intron 2, and the *DRB1* exon 2 locus whereby each *DRB1* exon 2 allele was in a 1:1 correspondence with an STR of unique size in the adjacent intron. However, in a subsequent study of a large population of Scottish Blackface sheep the 1:1 correspondence between exon 2 allele and STR size was not always present (Schwaiger *et al*., 1995). While the extent of the loss of 1:1 correspondence has been characterised in the Scottish Blackface breed, this finding casts doubts over the sole use of this allele prediction technique for *DRB1* exon 2 typing in other sheep breeds.

Using previously published *DRB1* exon 2 data from purebred Suffolk and Texel breeds (Sayers *et al*., 2005) this paper examines the 1:1 correspondence between the STR and *DRB1* exon 2, to investigate if STR analysis of *DRB1* intron 2 could be used to accurately assess the profile of *DRB1* exon 2 in these breeds.

Materials and Methods

DRB1 exon 2 genotyping

A total of 31 Suffolk and 60 Texel sheep were used in this study. Lambs were initially typed at the *DRB1* exon 2 locus, using a sequence-based-typing technique. This technique directly identifies the *DRB1* exon 2 allele sequence using PCR primers designed to flank the complete exon 2 region, as described in detail by

Sayers *et al*. (2005). The animals were chosen specifically to ensure that the alleles were represented at a reasonable frequency. Six and eight *DRB1* exon 2 alleles were identified in the Suffolk and Texel animals chosen for this study, respectively (Sayers *et al*., 2005).

Simple tandem repeat (STR) size analysis

Two methods, Genescan™ and autoradiography, were compared for STR size analysis. For the Genescan™ analysis, the *DRB1* exon 2 and adjacent STR were amplified by PCR as described previously (Schwaiger *et al*., 1995), substituting ^{32}P-CTP with a florescent 6-FAM-labelled forward primer (Sigma, UK). Approximately 1.5 ng of a 1:10 dilution of PCR product was incubated with 2.5 µl of deionised formamide and 1.5 µl of Genescan™-2500 TAMRA™ size standard (Product number 401545, Applied Biosystems, California, USA) at 90 °C for 2 min. The mixture was electrophoresed on an ABI Prism® 377 DNA sequencer for 5 h. The local Southern method was the algorithm of choice because it normalises data across lanes and gels (Genescan Reference Guide, 1997). Stutter fragments were identified for all samples, a phenomenon common in microsatellite amplification (Shinde *et al*., 2003). The interpretation of the correct allele length was standardised by choosing the peak at the far right position in all cases.

In the analysis by autoradiography, amplification of the *DRB1* exon 2 alleles was carried out in a 25-µl PCR reaction containing 100 ng DNA, 1 unit *Taq* polymerase (Promega, UK) and 0.2 µCi of ^{32}P-CTP under the following conditions: 1 min denaturation at 94 °C, 1 min annealing at 60 °C and 1 min extension at 72 °C for 30 cycles in a Perkin Elmer PCR cycler. The primers have been described previously (Schwaiger *et al*., 1995) and amplify exon 2 of the *DRB1* gene and the STR located in the second intron. Approximately 3 µl of PCR product was electrophoresed on a denaturing 6% polyacrylamide gel for 7 h at 60 W. The gel was exposed to X-ray film at –70 °C overnight and then developed. PCR band sizes were estimated by comparison to standards of known length, from the smallest size designated 'A', to the largest size designated 'M' (Schwaiger *et al*., 1995).

Results

Genescan™ technology takes advantage of proven multicolour fluorescence technology for sizing PCR fragments. This technology automatically identifies all PCR fragments and compares the mobility of each fragment to that of internal standards to calculate the fragment size.

Genescan™ analysis identified 8 STR sizes in the group of Suffolk animals investigated. Each allele was in a 1:1 correspondence with a specific STR size except for OAMHC213 and Ovar-*DRB*10 where associations with two STR's were observed (Table 1). STR size analysis using autoradiography identified a unique band for all alleles but did not distinguish between the 502 and 504 or between 572 and 574. Consequently, six band patterns were identified for eight different alleles using this technique, as shown in Table 1.

Genescan™ analysis identified 10 STR sizes in the group of Texel animals investigated. In this breed the 1:1 correspondence was not maintained for Ovar-*DRB1**0203 or Ovar-*DRB*10, as presented in Table 2. STR length analysis using autoradiography, identified eight band sizes but these bands did not correlate with the *DRB1* exon 2 alleles or the Genescan™ analysis (Table 2). The autoradiography

Table 1. The *DRB1* exon 2 allele, the STR sizes and the corresponding frequencies in Suffolk animals used in this study

Genebank accession no.	*DRB1*-exon 2 allele by sequence-based typing	STR size analysis by		Occurrence within selected population
		Genescan™ (bp)	Autoradiography (band size)†	
U00236	Ovar-DRB33	472	A	12
Y10246	OAMHC213	502	B	12
		504	B	3
AB017218	Ovar-*DRB1**03411	516	D	23
U00224	Ovar-DRB21	522	δ‡	4
AB017206	Ovar-*DRB1**0203	532	G	2
U00212	Ovar-DRBI0	572	M	2
		574	M	4

†'A' to 'M' represent band sizes from the smallest to the largest, respectively. These sizes are based on standards as used by Schwaiger *et al.* (1995).
‡This size band was slightly larger than the 'D' band but smaller than the 'E' band. It had not been previously identified. The 'E' band was not present in this Suffolk population.

method was unable to differentiate between the 507, 510 and 516 base pairs, the 532 and 536 base pairs or the 572 and 574 base pairs. A typical Genescan™ output is shown in Figure 1.

Ovar-*DRB1**0203 and Ovar-*DRB*10 were common to both breeds and maintained their STR association, 532 and 572/574, respectively. However, STR size 504 was associated with Ovar-*DRB*19 in Texels while also associated with OAMHC213 in Suffolks, and STR size 516 was associated with Ovar-*DRB1**03411 in Suffolks while also associated with Ovar-*DRB*29 in Texels.

Discussion

Genescan™ as a method of STR analysis gave a more reliable prediction of exon 2 allele than autoradiography. The expected 1:1 correspondence between the *DRB1* exon 2 allele and STR size in the adjacent intron, as described by Schwaiger *et al*. (1993), was not observed for all alleles in the animals examined in this study.

Table 2. The *DRB1* exon 2 allele, the STR sizes and the corresponding frequencies in Texel animals used in this study

Genebank accession no.	*DRB1*-exon 2 allele by sequence-based typing	STR size analysis		Occurrence within selected population
		Genescan™ (bp)	Autoradiography (band size)†	
U00222	Ovar-*DRB*19	504	B	13
AF036560	Ovar-*DRB1**0205	507	D	5
AB017209	Ovar-*DRB1**0109	510	C,D	22
U00232	Ovar-DRB29	516	D, δ‡	9
AY227049	Ovar-*DRB1**GS1	528	F	37
AB017206	Ovar-*DRB1**0203	532	G	4
		536	G	14
U00206	Ovar-DRB04	542	H	11
U00212	Ovar-DRB10	572	M	2
		574	M	3

†'A' to 'M' represent band sizes from the smallest to the largest, respectively. These sizes are based on standards as used by Schwaiger *et al.* (1995).
‡This size band was slightly larger than the 'D' band but smaller than the 'E' band. It had not been previously identified. The 'E' band was not present in this Texel population.

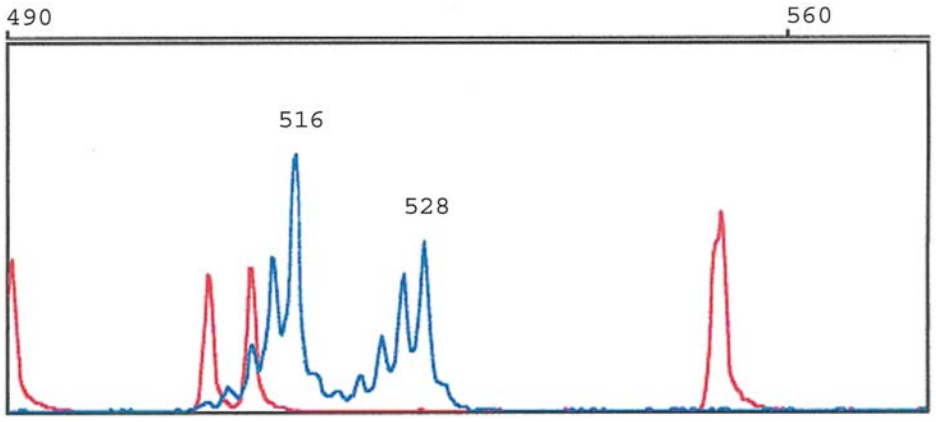

*Figure 1: This Genescan™ output represents a heterozygous Texel animal, with STR sizes 516 and 528, which correspond to allele Ovar-DRB29 and Ovar-DRB1*GS1, respectively. Taq DNA polymerase stuttering is evident for both fragments. Each stutter causes a 2-base pair decrease in length, representing one repeat unit. The right-most peak for each fragment was chosen as the correct STR size. Red peaks represent TAMRA™ standard length sizes, which were run with all samples.*

Two methods were used for STR size analysis in this study, autoradiography which has been used extensively in large animal studies (Schwaiger *et al*., 1995) and Genescan™, which was employed as a non-radioactive high-resolution alternative. The autoradiography method used here did not correlate with results from the Genescan™ analysis in Suffolks or Texels. In both breeds, STR analysis by autoradiography, was unable to differentiate between closely related STR sizes. Reasons for the inaccuracy include the lower resolution caused by radioactivity diffusion, *Taq* stuttering and a 'smiling' effect common to large electrophoresis gels. Genescan™ was unhindered by these drawbacks, because radioactivity was not used, the higher resolution differentiated stutter fragments and a migrating size standard was run with each sample, thus eliminating a 'smiling' problem. In addition, as 6-FAM and TAMRA™ differ strongly by their emissions, the sample and controls were easily distinguished.

A drawback when using microsatellite length for genotyping, is a stutter phenomenon that is the result of slippage by *Taq* DNA polymerase during PCR. The consequence of stuttering is a PCR product that shows slight variation in length, depending on the number of repeats in the microsatellite (Shinde *et al*., 2003). This phenomenon was more evident in Genescan™ analysis, because separation to one base pair resolution was possible as shown in Figure 1. *Taq* stuttering was present but not visible on the autoradiograph gel, because of the lower resolution caused by diffusion of the radioactivity.

While the 1:1 correspondence between *DRB1* exon 2 allele and STR size as determined by Genescan™ was upheld for the majority of alleles examined, in some situations two STRs were associated with one allele. Specifically, alleles Ovar-*DRB1**0203, Ovar-*DRB*10 and OAMHC213 were each in correspondence with two STR's. However, Genescan™ identified the correct allele because in

each case both STR's were unique to the allele in question. Accordingly, regardless of which STR size was identified by Genescan™, the correct allele was always assigned. However, employing the STR analysis by Genescan™ as a sole method of *DRB1* exon 2 genotyping in a *DRB1* exon 2 uncharacterised population is not reliable. On the basis of this study, genotyping by STR analysis alone would overestimate the allele profile by two *DRB1* exon 2 alleles in both breeds because of the described correspondence of three exon 2 alleles with two STR's each.

STR analysis by Genescan™ is potentially flawed for Suffolk × Texel crossbred progeny, because in some cases, some STR's will correspond to two different *DRB1* exon 2 alleles. For example, STR size 504 in a crossbred animal could correspond to either OAMHC213 or Ovar-*DRB*19. Similarly, STR size 516 in a crossbred animal will not differentiate between Ovar-*DRB1**03411 and Ovar-*DRB*29. If such an event occurs in a crossbred animal, a potential solution is to determine the correct exon 2 genotype by sequence-based typing, a task simplified because the STR analysis confines the allele genotype range to one of two alleles.

In the Suffolk population, described in this study, alleles Ovar-*DRB1**0203 and Ovar-*DRB*33 were associated with increased resistance while OAMHC213 and Ovar-*DRB*10 were associated with decreased resistance (Sayers *et al.,* 2005). Regardless of the STR analysis method employed, these important alleles would be correctly identified in a purebred Suffolk population flock. However in Suffolk × Texel crossbred progeny, alleles OAMHC213 and Ovar-*DRB*19 cannot be differentiated. Genescan™ is recommended rather than autoradiography for its ease of operation and lower cost.

In general terms, the linkage disequilibrium between the *DRB1* exon 2 alleles and specific STR's is not absolute, as proven by some *DRB1* exon 2 alleles in correspondence with two STR sizes. While its accuracy is proven in purebred Suffolk and Texels, the use of STR analysis as a *DRB1* exon 2 genotyping technique in other breeds, requires prior analysis of the correspondence between STR and *DRB1* exon 2 alleles. If its accuracy is proven within a population, STR analysis is recommended, because of its simplicity and speed.

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